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14. ABSTRACT

Four treatments were compared in an acute femur fracture model in swine for their capacity to improve the progress of bone healing and regeneration. The treatments examined were 1) salmon fibrinogen and thrombin, 2) porcine fibrinogen and thrombin, 3) bovine collagen and a commercial FDA approved treatment called CopiOs. The treatments were instilled into the injury site and the bone was stabilized by surgical external fixation. The subject animals were maintained for 3 weeks and examined biweekly using fluoroscopy to visualize the bone healing and Near Infrared Spectroscopy, electric impedance measurement and Doppler ultrasound to assess the healing of the sound tissue. At the end of three weeks, the animals were sacrificed and the injured limbs were recovered and examined by computer tomography. The femurs were processed for histology. Wound severity was consistent in 29 animals. Cellularity and bone regeneration varied by treatment with CopiOS being the least effective in inducing a growth response and salmon fibrinogen and porcine fibrinogen being the most inductive.

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Development of an Injectable Salmon Fibrinogen-Thrombin Matrix to Enhance Healing of Compound Fractures of Extremities

INTRODUCTION

Penetrating injuries to extremities caused by blast fragmentation include extensive tissue damage accompanied by uncontrolled bleeding and high grade open fractures [1, 2]. Bleeding at distal sites is controlled by tourniquet application but proximal wounds (ex. femoral) may be inaccessible. Serious bone injuries can be later treated by autologous bone grafts but bone grafts are subject to the possibility of serious side effects, such as morbidity and infection at the secondary site, pain and limitations for the supply of bone graft material. To attempt to overcome these limitations, new directions in the first response to penetrating wounds are required. Bleeding must be controlled by the direct application of a hemostatic agent deep into the wound at the site of the bleeding. Second, fragmented bone must be stabilized if the limb will be saved [3]. The approach of this project is to inject fibrin sealants directly into the wound to stop the bleeding and into the bone lesion to provide an environment that will sustain the cellular component of bone and encourage bone regeneration. This technique can also act as a medium that can support the growth of cells, either native or exogenous mesenchymal stem cells, that may be inserted into the site [4, 5]. The objective of this project is to develop a new injectable reagent that can form a matrix at the site of a bone fracture and enhance the preservation of bone tissue following injury. This treatment should permit successful surgical repair and accelerate subsequent healing. Specific parameters that will be examined will be 1) cell viability of bone fragments at the site of injury as well as those embedded in the injected matrix but removed from contact with the primary bone, 2) resistance of the injured bone to necrosis, 3) induction of new bone growth at the site of injury and 4) comparison of rate of bone regeneration in animals treated with different implantable matrix. The material to be tested in this project is a salmon fibrin matrix derived from salmon fibrinogen and thrombin [6-8].

BODY

The methodology of this project is to create a fracture in the femur of pigs that will replicate the injuries caused by high velocity penetrating projectiles. The fracture is then stabilized by external fixation according to standard practice and the treatment agent is injected into the wound site. The limb is then further stabilized by the application of a fiberglass cast. The animals receive Fentanyl patches the day before surgery to initiate analgesia and continue to wear the patches for 2 weeks following surgery. Buprenorphine and metacam (Meloxicam) are also administered at the time of surgery for short term pain relief. Fluoroscopy is performed before and after injury to ascertain the degree of injury and to assess the reproducibility of the injury. Blood is drawn to permit assays for immune reaction and infection. Physiological monitoring of

the animal to measure vital signs, blood flow and edema in the hind legs is conducted to track changes following injury. Monitoring is continued every three days to include bandage change, fluoroscopy and physiological parameters. At three weeks the animals are sacrificed and the femurs are recovered for analysis. The bones are imaged using X-ray computerized tomography. Following imaging, the specimens are fixed in formaldehyde and processed for histological examination. Expert analysis of the histological specimens is provided by MAJ Eric Lombardini, veterinary pathologist.

In vitro studies are conducted using mesenchymal stem cells in culture. The cells are cultured in media with and without the treatment agents and assessed for cell viability and induction of osteogenesis. The osteogenesis is measured by cell staining and examination for morphological changes in the cells and by RNA analysis. Using real time PCR, the RNA expression is analyzed to determine if the different treatments have up-regulated the expression of bone-specific genes.

Changes to the methodology:

1. Surgery site care: The pin sites where the surgical bone pins (bone screws) pierce the skin have reproducibly become infected. Cultures of the infections have revealed a wide spectrum of organisms ranging from common skin infections to farm animal associated organisms such as glanders. We instituted cleaning of the pins sites every 2-3 days to combat this problem. It is very labor intensive since it involves anesthetizing the animal to do this. It has resulted in significantly reducing the frequency and extent of infection. However, infection can seriously impact the healing process and make interpretation of the results difficult (more in discussion of results).

2. Analgesia by nerve blockade. In conjunction with MAJ Joseph Royal DVM, USUHS, we have instituted a study of the efficacy of injection of an anesthetic agent directly to the site of the sciatic and femoral nerves. The sites are imaged using ultrasound and 10 mL of agent is injected into the site. Animals are randomized to receive active agent or buffer. Responses are assessed by direct observation and ECG monitoring (See below).

3. Remote scanning of electrocardiogram tracings to measure behavioral responses. We have in our possession the necessary instrumentation to remotely monitor the heart rate and ECG of the animals post-surgery. It has been suggested by conversations with colleagues working in the field that the behavioral response of the animals and resumption of normal activity may be influenced by the agent used for bone healing (ex salmon fibrinogen vs CopiOS). This can be evaluated by analysis of heart rate variability. Since this is a non-invasive technique utilizing equipment in our possession, this could be an added value source of data concerning the effects of the treatment that could be gathered at relatively low cost.

Progress in the project

In the **first year** of this project we have made progress on both the cell culture aspects of the project and the animal studies. By establishing stem cell cultures that have the capacity to differentiate into bone-producing cells, we have demonstrated that, with controlled culture conditions, we can manipulate the expression of bone-specific proteins. The expression of these proteins can be visualized by microscopic examination of the cells and by biochemical detection of bone-specific RNA. The expression levels can be modulated by culturing the cells with different matrices. We found that the salmon fibrinogen and thrombin matrix induced increases in 21 proteins associated with osteogenesis. Furthermore, these changes were dependent on the concentration of the salmon proteins in matrix. For the *in vivo* aspects of the project, we have established the basic femur fracture model and determined an efficient and humane method for caring for the animals post-operatively. We have treated 9 animals with either salmon fibrinogen, porcine fibrinogen,unmodified collagen and or TR Matrix. The bones have been CT scanned and prepared for histological examination. One set of salmon fibrinogen-treated bone fracture slides have been prepared and examined. Blood samples have been collected and basic blood parameters measured and evaluated for the production of adverse immune reaction.

In the **second year**, all four treatments were tested. These treatments were 1) salmon fibrinogen and salmon thrombin, 2) porcine fibrinogen and porcine thrombin, 3) a commercially available, FDA approved bone matrix called CopiOS and 4) bovine collagen. Our plan was to have four groups of eight animals. To date we have collected data from 29 animals. Several animals have been excluded from the data group due to termination prior to the completion of the trial period. Data collected included biological samples for immunology testing, physiological parameters to assess wound healing, fluorographs (X-rays) to assess the bone formation and growth and CT (computer tomography) for 3-D reconstruction of the bone. In addition, all bones were preserved for histological examination by Dr. Eric Lombardini, veterinary pathologist at AFRII. Our final results are still being analyzed because the last of the animals was only recently completed. However, preliminary results show that there are definite differences between animals, both at the level of radiographic examination and histological examination. The analysis of the radiographs is quantifying the degree of displacement of the bone, severity of injury and degree of fibrosis and calcification of the injury site. Sepsis at the bone fixation site and the injury site is also evaluated since this may have an impact on the healing process. Numerical scores are assigned to each parameter to permit comparison between samples and groups. The histological slides are assessed in a similar fashion with numerical score assigned to infection, bone formation and

osteoclast frequency. It will not be possible to make conclusions on the virtues of one treatment group versus another until we have analyzed all of the histological data.

A final aspect of the project was a subproject concerning the effectiveness of the pain alleviation protocol that was used. Dr. Joseph Royal, veterinary fellow, has conducted this project in which he administered femoral and sciatic nerve blockades in addition to the fentanyl patches that were the standard of care for the animal. These data are currently being analyzed for heart rate variability and activity levels which have been used as criteria for pain in other studies.

Initial analysis of in vivo data

Fluoroscopy

The fracture healing was assessed at three weeks (initially animals were assessed at 2 weeks, asterisk) for the following parameters:

KEY						
Category	Title	1	2	3	4	5
A	**Wound Severity** Degree of fragmentation associated w/loss of blood supply & delay healing	Only cortex is broken w/o discontinuity	Fracture of both Cortex w/o discontinuity or single frx	Fragments Partially aligned or partially displaced	(3+) multiple fragments w/ moderate displacement	(3+) multiple fragments not in contact, severely displaced
B	**Fx Stability Score** degree of commutation/ displacement associated w/increased callus formation but incomplete healing	Cortex is broken but no discontinuity	Discontinuity w/ good apposition	Discontinuity with partial apposition Presence of rotation, mal-alignment	Discontinuity w/moderate rotation mal-alignment, collapse	Complete displacement, rotation or mal-alignment
C	**Callus Proliferation Along Fracture** (periosteal endosteal)	No presence of fibrous or calcified callus	Presence of slight thickening of fracture margins	Moderate presence of callus w/bridging-internal and external	Contacted callus between fractures but not joined-visible Fx line	Complete bridging along fracture(s) with calcification present
D	**Wound Fill Score** Proliferation at injury site	No presence of fibrous or calcified	Slight presence of F/C callus	Moderate F/C callus	Moderate filling of void w/endosteal callus formation	Complete filling of wound w/calcification
E	**Infection Score**	No presence of infection	Infection limited to pin site only	Slight infection extending beyond pin site	Moderate osteomyelitis not extending to wound	Infection extended to wound

Based on the parameters explained above, fluoroscopy of each animal's femur was examined and graded. Scores were graded by consensus between the two primary investigators. The investigators were blinded as to the treatment used until after the scoring was complete. Treatment code: P = porcine fibrinogen/thrombin; S= salmon fibrinogen/thrombin; B = bovine collagen prepared in the investigator lab and C = CopiOS/T= TRmatrix. The last two are commercially prepared bone matrix fillers based on calcium phosphate and bovine collagen. CopiOS is an FDA approved treatment for human injury.

Table 1 Scoring of bone healing by fluoroscopy:

Treatment	Animal #	Wound Fill	Fracture Callus	Wound Severity	Fracture Stability	Infection
P	28201	4.5	5	2	2	2
P	30420	4.5	4	4	4	2
S	30435	4.5	3.5	3.5	4	1.5
S	28096	4	5	2.5	2.5	4
B	28655	4	4.5	3.5	3.5	2
P	29711	4	4.5	3.5	2.5	3
S	27874	4	4	3	3	4
P	28441	4	4	2	4	2
B	28654	4	4	3	4	3
B	30568	4	4	3.5	3.5	2.5
P	29712	4	3	3	4	4.5
P	30118	4	3	3.5	4	2
B	31270	4	3	1.5	3	4
B	30567	4	2	3	1.5	2
S	28651	3.5	4	3	2	2
C	29707	3.5	4	3	2.5	2
C	30274	3.5	3	3	3	1
C	31200	3.5	3	3.5	4	4
S	30117	3	4.5	3.5	2	2
C	29704	3	4	3.5	4	4.5
S	31272	3	3	3	3.5	4.5
S	28439	3	2.5	1	1	2
T	28095	2.5	2	3	2	2
C	29228	2	4	2	2	4.5
S	30461	2	3.5	3	3	5
P	28200	2	3	4	4	2
C	29229	2	3	4.5	3.5	3
C	30455	2	2.5	4	5	2
B	28044	1	2	3	3	5

The animals are ranked in Table 1 by wound fill, with 5 being the most filled-in wound and 1 being the least. Although the analysis is still being refined, with the extra parameter of time of healing yet to be added in, there are several initial conclusions that may be drawn. The first is that the commercial bone fillers consistently ranked at the bottom of the groups. The second conclusion is that, generally, the higher the infection scores, the lower the healing or filling score. This is despite the fact that none of the actual wound sites became infected. The infection started at the fixation pin sites and would spread into the bone. If the infection spread sufficiently, it would impact on the healing process. The third conclusion is that the biological fillers were approximately equivalent in this small sample size with the top half of the wound filling treatments being equally distributed among the biological fillers (fibrinogen and collagen vs the calcium phosphate fillers)

Histology

Histology of the bone healing was graded by visual examination of the pathologist, Dr. Eric Lomdardini, DVM, AFRRI, based on the criteria below.

HISTOPATHOLOGIC DIAGNOSES KEY

Severity scoring based on inflammation scale 1-5 (minimal, mild, moderate, marked, severe)

Maturation scoring based on fibroblast infiltration, collagen deposition and myelofibrosis combined on a scale from 1-5 (1=most immature to 5=most mature)

New bone growth is scored qualitatively on a 1-5 scale

Osteoclasts/HPF are averaged over 10 high powered fields

Table 2 Histology scoring of bone growth

Treatment	Pig ID#	Severity Scoring	Maturity Scoring	New bone growth	Osteoclasts /HPF
B	**10-1439: 28044**	5	5	3	7
B	**10-1471: 28654**	3	2	1	1
B	**10-1472: 28655**	1	3	2	3
B	**11-1216 (P30568)**	2	3	2	1
B	**11-1217 (P30567)**	4	3	3	4
B	**11-1389 (31270)**	4	3	3	6
C	**11-003: 29229**	3	3	3	3
C	**11-005: 29228**	1	5	5	2
C	**11-1018 (29704)**	3	3	2	<1/10 HPF

C	**11-1019 (29707)**	3	2	4	10/HPF (regionally)
C	**11-1215 (P30274)**	2	4	2	1
C	**11-1385 (30455)**	3	5	4	4
C	**11-1583 (31200)**	5	2	4	<1
P	**10-1437: 28200**	2	3	5	4
P	**10-1441: 28441**	5	5	2	2
P	**10-1470: 28201**	2	1	1	1
P	**11-403: 29711**	5	4	5	8
P	**11-404: 29712**	5	5	2	6
P	**11-1214 (P30118)**	3	3	2	<1/10 HPF
P	**11-1387 (30420)**	2	5	3	1
S	**10-1124: 27874**	4	3	4	3
S	**10-1436: 28096**	5	4	2	1
S	**10-1438: 28439**	5	4	5	8
S	**10-1473: 28651**	3	2	3	2
S	**11-402: 29531**	5	2 (regionally 3)	5	2
S	**11-1386 (30461)**	5	5	5	1
S	**11-1388 (30435)**	3	4	4	1
S	**11-1584 (31272)**	5	2	2	<1
T	**10-1440: 28095**	1	5	1	1

The interpretation of the histology results is still being considered but using the new bone growth scores, the salmon fibrinogen was somewhat better than the other three treatments

Table 3 Comparison of mean bone growth

Treatment	Salmon fibrinogen	Porcine fibrinogen	Bovine collagen	copiOs
New growth	3.75	2.86	2.33	3.42

Analysis of physiology data and pain assessment

The electronic collection of data generated large amounts of that that must be manually curated. This process is still on-going.

Problems encountered this year

At the beginning of year two, the primary technician on this project was offered a new position at a biotech firm, which she accepted. Despite the economic conditions, it took a number of months to hire a new permanent technician. In the absence of a technician, the PIs ended up doing most of the animal work themselves. A new technician is now settled into the project and it is anticipated the rest of the items on the project list will be soon be completed.

KEY RESEARCH ACCOMPLISHMENTS

- Completed the full schedule of in vivo testing of the four treatment groups for enhanced bone healing.
- Completed the computerized tomography scanning of all fractured femur.
- Completed the fluoroscopic imaging of all injury sites.
- Completed the histology preparation of all fractured.
- Completed the digitization of all histology slides.
- Collected cardiac tracings from twenty animals treated with regional nerve blocks.

Personnel receiving salary from this research project (Year 2)

Dr. Michael Bodo, research physiologist

REPORTABLE OUTCOMES

- Presented poster on hemostatic qualities of salmon fibrinogen and thrombin at the Advanced Technology Applications For Combat Casualty Care 2011
  S. W. Rothwell, C. Timothy Floyd. 2011. A Salmon Thrombin-Fibrinogen Dressing Controls Hemorrhage in a Swine Model Compared to Standard Kaolin-Coated Gauze. Advanced Technology Applications for Combat Casualty Care, St Pete Beach, FL.

- Preparing poster on the comparison of bone healing properties to be presented at the Special Operations Medicine Association meeting in December 2011 in Tampa, FL

CONCLUSIONS

In summary, we have made substantial progress in achieving the goals outlined in our project proposal. We have collected the data from all of our animals and have made initial analysis and conclusions. We are developing a computerized analysis methodology to quantitate the area of bone calcification and validate the observational measurements of the Dr. Lombardini. The animals recovered well from the fibrinogen treatment. In comparison, by subjective observation, the animals with the collagen treatment did not recover normal levels of activity as well with those treated with the fibrinogen treatments. We are now doing the heart rate variability analysis to determine if this analysis correlates with the subjective observations. We are also using ELISA to determine the level of analgesic drugs and cytokine levels to determine if the drug levels correlate with our estimated pain levels. Other on-going work includes completion of the immunological analysis of the plasma and serum, assessment of the coagulation status and analysis of the physiological data.

It appears that there may not be a clear and absolute winner among the treatments by statistical criteria, but the fibrinogen treatments seem to be better than the collagen and collagen and Ca/P treatments in these preliminary analyses. Based on our experience with this pig model, we would like to extend this study in our next project in a small animal model with larger numbers. In that case, the animal would fit into the CT scanner while anesthetized so that healing can be followed longitudinally over a longer period of time. We would also avoid external fixation to reduce likelihood of infection.

Based on the findings from Year 2, we are optimistic that the salmon fibrinogen/thrombin treatment can be a useful adjunct treatment in severe bone injury. Our experience with this treatment in hemorrhage models has demonstrated that the salmon treatments are very effective in stopping bleeding. This was also observed in this project. The salmon treatment is currently under commercial development as a hemostatic device by the biotech company, St,Teresa Medical. It would be a logical extension of the material's use to apply it to bone injury treatment.

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